Prevention of Alterations in Intestinal Permeability Is Involved in Zinc Inhibition of Acute Ethanol-Induced Liver Damage in Mice

JASON C. LAMBERT, ZHANXIANG ZHOU, LIPENG WANG, ZHENYUAN SONG, CRAIG J. MCCLAIN, and Y. JAMES KANG

Departments of Pharmacology and Toxicology (J.C.L., C.J.M., Y.J.K.) and Medicine (Z.Z., L.W., Z.S., C.J.M., Y.J.K.), University of Louisville School of Medicine, Louisville, Kentucky; and Jewish Hospital Heart and Lung Institute, Louisville, Kentucky (Y.J.K.)

Received December 9, 2002; accepted February 21, 2003

ABSTRACT

Acute ethanol exposure causes liver injury in experimental animals, and accumulating evidence suggests that a major responsible factor for the pathogenesis is endotoxemia, which results from bacterial endotoxin leakage from the small intestine due to increased intestinal permeability under alcohol challenge. The purpose of this study was to examine whether zinc pretreatment would inhibit acute ethanol-induced liver injury through prevention of intestinal permeability changes. Male 129 SvPCJ mice were treated with three intragastric doses of ZnSO4 at 5 mg of zinc ion per kg each dosing prior to acute ethanol challenge with a single oral dose of 6 g/kg ethanol. The zinc treatment did not alter the elevation of serum concentrations of alcohol. The acute ethanol exposure caused an elevation in serum alanine aminotransferase levels as well as fatty liver and hepatic degenerative necrotic foci as determined by biochemical assay and histochemical analysis, respectively. A significant increase in liver tumor necrosis factor-α (TNF-α) levels was detected by enzyme-linked immunosorbent assay. These pathological effects correlated well with increases in serum endotoxin levels. Importantly, acute ethanol treatment caused significant damage to the small intestine as determined by morphological analysis of intestinal sections and permeability assay. These alcohol-induced hepatic pathological changes and TNF-α elevation were significantly inhibited in the zinc-pretreated animals. The inhibitory action of zinc on alcohol-induced liver damage and activation of inflammation was associated with zinc suppression of alcohol-induced intestinal permeability changes. These results thus demonstrate that zinc prevention of increased intestinal permeability is importantly involved in the inhibition of acute ethanol-induced liver damage in mice.

Acute ethanol exposure causes significant damage in the livers of mice, with common pathological changes observed, including microvesicular steatosis, apoptosis, necrosis, and inflammation (Zhou et al., 2001, 2002). Importantly, increasing evidence implicates endotoxemia as a primary cofactor in the induction of acute ethanol-induced liver pathogenesis (Enomoto et al., 1998; Rivera et al., 1998). Endotoxins are glycolipid components of the outer wall of Gram-negative bacteria that colonize the large and small intestine (Su, 2002). Prior studies have shown that livers from rats acutely exposed to ethanol experienced a transient increase in plasma endotoxin leading to liver damage (Shibayama et al., 1991; Enomoto et al., 1998). Furthermore, sterilization of the intestinal system with antibiotics prior to ethanol challenge leads to a significant decrease in liver damage of rats, implicating the critical involvement of bacterial endotoxins in alcohol-induced liver injury (Adachi et al., 1995).

Endotoxin is cleared from the circulation by Kupffer cells, the resident macrophages of the liver (Saba, 1970). In response to endotoxin, Kupffer cells produce proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, and reactive oxygen species that are toxic to the surrounding parenchyma (Arthur et al., 1988; Luster et al., 1994). Injection of anti-TNF antibodies as well as pentoxifylline, an inhibitor of TNF-α secretion, into animals has been shown to protect the liver (Tracey et al., 1987; Bachmann et al., 1992). In addition, Kupffer cells have been illustrated to play an important role in hypoxia reoxygenation-induced damage of liver parenchyma following acute ethanol exposure, through initiating a swift increase in alcohol metabolism via secretion of prostaglandins (Rivera et al., 1998). Inactivation of Kupffer cells with GdCl3 in vivo leads to a decrease in liver damage and improves mortality following ethanol treatment in experimental animals (Adachi et al., 1994). Collectively, these data implicate Kupffer cells as an important cofactor in ethanol-induced liver injury.

Under normal physiological conditions, the intestinal mu-
costal layer allows small antigens and macromolecules to pass through in small quantity (Bode, 1990). However, acute ethanol exposure increases the permeability of the small intestine to larger macromolecules, including bacterial endotoxins (Tamai et al., 2000). The specific mechanisms involved in elevation of intestinal permeability to endotoxin during alcohol exposure remain unclear. However, it has been shown that the effects of acute treatment with ethanol on intestinal membrane structure are dose- and time-dependent, and the damage incurred by different sections of the small intestine appears to be related to proximity to the gastric compartment (Beck and Dinda, 1981).

Previous studies have described that as many as $2.4 \times 10^8$ bacteria per gram of tissue reside in normal rat small intestine (Yi et al., 1999). These intestinal bacteria “turnover” on a daily basis and as a result release cell wall components, creating an “endotoxin-rich” environment in the lumen of the gut. Therefore, increased permeability of the intestine may allow excessive levels of endogenous bacterial endotoxin to leak into portal blood flow, causing liver injury. The support for this speculation was that rats treated acutely with ethanol experienced an increase in plasma endotoxin and that this was a function of increased intestinal absorption from the small intestine (Tamai et al., 2000). Thus, it is imperative that protection of the liver from the deleterious effects of ethanol mediated by endotoxin should be developed at the level of intestinal mucosa. Recent studies showed that dietary supplementation of rats with oats protected the liver from chronic alcohol injury by preventing intestinal leakiness and endotoxemia; however, this result was perplexing because intestinal injury was not significantly different in the oats-treated rats challenged with ethanol versus the ethanol-treated controls (Keshavarzian et al., 2001). Our recent studies have shown that zinc supplementation of mice prior to acute ethanol challenge significantly protected the liver from injury (Zhou et al., 2002). This zinc protection was shown to relate to antioxidant action in hepatocytes. However, zinc supplementation has proven to be protective in many intestinal disease processes that involve changes in permeability, including chronic inflammatory bowel disease or Crohn’s disease and acute shigellosis (Alam et al., 1994; Sturmiolo et al., 2001). Therefore, we hypothesized that zinc supplementation would ameliorate acute ethanol-induced liver injury by preventing an increase in intestinal permeability and subsequent endotoxemia.

Materials and Methods

**Animals.** Male 129/SvEvTac mice (20–25 g, 8-10 weeks of age; The Jackson Laboratory, Bar Harbor, ME) were used in this study. They were maintained at 22°C with a 12-h light/dark cycle and had free access to rodent chow and water. The experimental procedures were approved by the Institutional Animal Care and Use Committee, which is certified by the American Association for Accreditation of Laboratory Animal Care.

**Acute Ethanol Challenge.** A binge drinking model developed by Carson and Pruett (1996) was followed for acute ethanol challenge. This model was designed to achieve blood alcohol levels that would produce physiological effects comparable with human binge drinking. Animals were divided into four treatment groups (ten mice per group) in a $2 \times 2$ factorial design (+/- zinc, +/− ethanol): 1) isocaloric maltose water control, 2) ethanol, 3) zinc, and 4) zinc plus ethanol. In groups 3 and 4, mice were given ZnSO4 (Sigma-Aldrich, St. Louis, MO) intragastrically at a dose of 5 mg of zinc ion/kg b.wt., in 12-h intervals for 24 h. In groups 1 and 2, mice were given sterile saline intragastrically as a vehicle control. Mice were fasted for 16 h, and mice in groups 3 and 4 then received the third and final dose of zinc. After 1 h, in groups 2 and 4, mice were administered ethanol (30% w/v) (Aldrich Chemical Co., Milwaukee, WI) in a single oral dose of 6 g/kg b.wt. by gavage, and group 1 and 3 mice received isocaloric maltose water by gavage on the same schedule. The time of necropsy for determination of plasma endotoxin levels and analysis of the ileum was 1.5 h following ethanol or maltose water administration. For examination of liver TNF-α, liver histology, and serum enzymes, the time of necropsy was 6 h following ethanol or maltose water. Mice were anesthetized with sodium pentobarbital (0.05 mg/g b.wt.) (Abbott Laboratories, North Chicago, IL). Blood was drawn from the dorsal vena cava, livers were perfused and harvested, and 1.0-cm sections of the duodenum, jejunum, and ileum were obtained for analysis. Stored tissues were first flash frozen in liquid nitrogen and then placed in −80°C until analysis.

**Blood Alcohol Assay.** Serum blood alcohol levels were measured using an alcohol dehydrogenase kit (procedure 332-UV; Sigma-Aldrich) according to the instructions of the manufacturer.

**Alanine Aminotransferase Assay.** Serum alanine aminotransferase (ALT; EC 2.6.1.2.) activity was colorimetrically measured using a diagnostic kit (procedure 505; Sigma-Aldrich) according to the instructions of the manufacturer.

**Histopathological Examination of the Liver and Intestinal Sections.** Liver and intestinal histological slides were prepared as described previously (Zhou et al., 2002), and hematoxylin and eosin (according to Ehrlich; Fluka, Milwaukee, WI) staining of liver and intestinal sections were resolved by light microscopy.

**Plasma Endotoxin Assay.** Blood samples from control and treated mice were drawn from the dorsal vena cava via sterile heparinized syringes. Platelet-rich plasma was obtained by centrifuging the whole blood at 300 g for 15 min at 4°C. Plasma samples were diluted 1:10 with sterile nanopure water, mixed by vortex, and placed in a 75°C water bath for 10 min. Samples were allowed to cool to room temperature for 10 min prior to colorimetric assay using the limulus amebocyte lysate (LAL) kit (BioWhittaker, Walkersville, MD). Standards and samples were incubated with LAL for 10 min at 37°C followed by 6-min incubation with colorimetric substrate. The reaction was stopped with 25% acetic acid, and the absorbance was read in a microplate reader at 405 nm.

**Isolation of Liver TNF-α.** Liver pieces (0.75–1.0 g) were minced thoroughly in ice-cold radioimmunoprecipitation assay buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris base, 0.3% Triton X-100, 0.03% sodium dodecyl sulfate, 0.3% Na-deoxycholate, and 1% protease inhibitor cocktail, pH 7.4) followed by incubation on ice for 30 min. The homogenates were then centrifuged at 15,000g for 20 min at 4°C. The supernatants were removed to clean tubes and centrifuged again at 15,000g for 20 min at 4°C. The supernatants of this spin were then used for enzyme-linked immunosorbent assay (kit no. KMC3012; BioSource International, Camarillo, CA).

**Intestinal Permeability Assay.** To evaluate the effect of ethanol on intestinal permeability, bacterial lipopolysaccharide (LPS; Escherichia coli serotype 0111:B4; Sigma-Aldrich) leakage from the intestine by examining blood levels of LPS after intestinal loading was applied. To determine an appropriate loading dose of exogenous LPS, we performed a dose response study by administering mice different concentrations of LPS by gavage. Thirty minutes after loading with LPS, the animals were sacrificed, and blood was collected for analysis by endotoxin assay. After the dose-response study, a dose of 1 mg/kg b.wt. LPS was chosen and given to the mice 1 h after the treatment with isocaloric maltose water or ethanol, and the same procedure was followed to determine the blood endotoxin levels.

**Statistics.** Data were expressed as mean ± S.D. (n = 6–9) and analyzed according to a $2 \times 2$ (zinc versus ethanol) factorial experimental design. After a significant interaction was detected by the two-way analysis of variance, the significance of the main effects was
further determined. The level of significance was considered at $P < 0.05$.

**Results**

**Blood Alcohol Level (BAL).** At 1.5 h after ethanol administration by gavage, the BALs in ethanol-treated mice were measured as shown in Fig. 1. Alcohol treatment significantly increased BALs, and zinc pretreatment did not change this elevation. This result thus indicates that zinc pretreatment did not affect alcohol absorption and that any observed protective effects of zinc in the liver and small intestine were not due to a decreased level of exposure to ethanol.

**Inhibition of Acute Ethanol-Induced Liver Injury by Zinc Pretreatment.** Oral administration of alcohol caused marked liver injury as examined by serum markers of liver damage and hepatic histopathological changes. As shown in Fig. 2, acute ethanol exposure caused a 4-fold increase in the levels of serum ALT and AST compared with control animals. Pretreatment of mice with zinc prevented the elevation of serum ALT and AST, thus indicating the protective effect of zinc on acute ethanol-induced liver injury. The most common histopathological change observed in the liver following acute ethanol treatment is microvesicular steatosis, as seen by the engorgement of hepatocytes with fatty vesicles in the cytosolic compartment (Fig. 3C). In addition, there was mild necrosis found in the livers of acute ethanol-treated mice, which is characterized as enlarged hepatocytes with faint or absent nuclei (Fig. 3C). The livers of control and zinc-treated mice appeared healthy, with no observable anomalies (Fig. 3A and 3B). Zinc pretreatment significantly inhibited the steatosis and necrosis in the liver, as seen by the diminished fatty infiltration of hepatocytes and no observable necrosis (Fig. 3D).

**Inhibition of Acute Ethanol-Induced Liver TNF-α Production.** Acute ethanol exposure elicited a 4-fold increase in liver TNF-α levels as compared with control animals (Fig. 4). Zinc-treated animals had levels of liver TNF-α approximately equal to that of untreated controls and mice treated with zinc prior to acute ethanol exhibited significant inhibition ($p < 0.05$) to the increase in the liver TNF-α levels (Fig. 4).

**Inhibition of Acute Ethanol-Induced Endotoxemia.** As illustrated in Fig. 5, acute ethanol exposure caused a 6-fold increase in the level of plasma endotoxin compared with control mice. Zinc pretreatment significantly ($p < 0.01$) inhibited this ethanol-induced effect where the concentration of plasma endotoxin was only slightly elevated compared with zinc-treated control animals.

**Prevention of Acute Ethanol-Induced Histopathological Changes in the Intestine by Zinc Pretreatment.** Acute ethanol exposure caused significant injury to the ileal small intestine of mice. There was severe injury to the mucosal lining, with breaches in the epithelial layer of the villi (Fig. 6C). In addition, submucosal blebbing and ulceration of villi were observed (Fig. 6C). In contrast, zinc-pretreated mice suffered no observable alterations in the ileum following acute ethanol challenge (Fig. 6D). The villi appeared uniform with no blebbing or loss of epithelial cells.

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**Fig. 1.** Blood alcohol levels measured from sera obtained 1.5 h after intragastric administration of 6 g/kg alcohol (EtOH; $n = 9$). Controls were treated by the same route with isocaloric maltose water (Con; $n = 7$). Some mice were treated with 5 mg/kg ZnSO$_4$ three times in 12-h intervals and administrated with isocaloric maltose water 1 h after the last dose of zinc (Zn; $n = 7$), and others were pretreated with zinc followed by ethanol challenge 1 h after the last zinc administration (Zn + EtOH; $n = 9$). Results are means ± S.D. *, significantly different from Con group.

**Fig. 2.** Serum ALT and AST levels 6 h after intragastric administration of alcohol. These mice were administered 6 g/kg alcohol by gavage (EtOH; $n = 8$), and an equivalent volume of isocaloric maltose water was given to controls (Con; $n = 6$). Some mice were treated with 5 mg/kg ZnSO$_4$ three times in 12-h intervals followed by isocaloric maltose water treatment as described for Fig. 1 (Zn; $n = 6$), and others were pretreated with zinc followed by ethanol challenge 1 h after the last zinc administration (Zn + EtOH; $n = 8$). The serum ALT and AST activities were determined by colorimetric assay. Results are means ± S.D. *, significantly different from Con group.
Acute Ethanol-Induced Intestinal Permeability Changes. Permeability alterations of mouse intestine due to ethanol were determined by measuring LPS leakage from the intestine 30 min after intragastric loading of exogenous LPS. To define an optimal dose of LPS for the test, different LPS concentrations of 1, 2, 5, and 10 mg/kg were loaded intragastrically. The results showed that an LPS dose of 2 mg/kg or less produced blood levels of endotoxin that were approximately equal to that observed in the control animals. However, LPS doses above 5 mg/kg produced a 5- to 6-fold increase in the detectable blood endotoxin levels. Thus, a dose of 1 mg/kg was chosen to determine the effect of ethanol on intestinal permeability.

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Kupffer cells release various cytokines, such as TNF-α and interleukins, as well as prostaglandins and reactive oxygen radicals (Martinez et al., 1992; Luster et al., 1994; Enomoto et al., 1998). Previous studies have illustrated that alcohol administration increases circulating TNF-α levels and that down-regulating TNF-α expression or administering anti-TNF-α antibodies attenuated alcohol-induced liver injury (Honchel et al., 1990; Kamimura and Tsukamoto, 1995; Iimuro et al., 1997). It is well known that hepatocytes are rich in high-affinity, low-capacity tumor necrosis factor receptors, rendering them highly sensitive to TNF-α (Deaciuc et al., 1995). Therefore, TNF-α secreted by activated Kupffer cells has a significant direct toxic effect on the surrounding parenchyma. In this study, we observed a significant increase in the liver TNF-α levels 6 h after ethanol exposure, and this increase was significantly depressed in the zinc-pretreated animals. This result suggested that zinc prevention of a Kupffer cell-mediated inflammatory response is critical in protecting the liver from acute ethanol-induced injury.

Rats treated acutely with ethanol have been shown to experience a transient increase in plasma endotoxin, with maximal levels observed 1 h following administration (Enomoto et al., 1998). Adachi et al. (1995) showed that sterilization of the intestine of rats with antibiotics caused a decrease in plasma endotoxin, and this result was associated with a significant inhibition of alcohol-induced liver injury. In rodent models of lethal shock, the mortality rate of mice challenged with an intraperitoneal injection of endotoxin was 100%, and this effect was almost completely abolished when mice were pretreated with zinc and 97% of the animals survived (Snyder and Walker, 1976). To determine whether zinc protection from acute alcohol-induced liver injury is through inhibition of endotoxemia, we measured plasma endotoxin 1.5 h after ethanol exposure, which has been shown previously to be in the peak time range for alcohol-induced endotoxemia (Rivera et al., 1998). We observed a 6-fold increase in plasma endotoxin of mice treated acutely with ethanol as compared with control mice, and zinc pretreatment almost completely blocked this ethanol-induced effect. It should be noted that we observed basal levels of plasma endotoxin in control (13.1 ± 6.6 pg/ml) and zinc-treated (14.5 ± 5.8 pg/ml) mice. Many previous studies reported near zero or undetectable levels of endotoxin in control animals (Rivera et al., 1998; Keshavarzian et al., 2001). However, it has been discussed in a recent study that there are a number of reasons for high baseline LPS levels in animal models of alcoholic liver disease in control animals (Mathurin et al., 2000). We were not able to define specific causes for the high basal levels of LPS in our control animals, although this stable high basal level of LPS has been consistently observed in our studies.

Acute ethanol-induced endotoxemia is hypothesized to be a result of increased intestinal permeability to endotoxin. Reports of ethanol-induced increases in intestinal permeability in the absence of morphological damage are common (Worthington et al., 1978; Keshavarzian et al., 1994). However, our work differed from many of the earlier acute ethanol studies. First, we looked at intestinal morphology 1.5 h after acute ethanol administration, when the levels of plasma endotoxin measured were maximal. In contrast, the time points for harvesting intestinal tissue for histochemical analysis in
other studies were from several hours to several days following acute ethanol exposure (Worthington et al., 1978; Keshavarzian et al., 1994). It is imperative to examine tissue before repair has taken place, and clearly the intestine is capable of restoring both morphological alterations and decreases in barrier function during recovery from alcohol challenge (Miljan et al., 1980; Tamai et al., 2000). Ileal intestine from mice treated acutely with ethanol in our study suffered gross morphological anomalies, where many intestinal villi had breaches in the epithelial layer. Zinc pretreatment profoundly protected the ileum from this acute ethanol-induced morphological phenomenon. Examination of zinc and ethanol-treated ileal sections showed normal villi with intact epithelial cell layers and no detectable abnormalities.

Second, determination of intestinal permeability in various ethanol studies ex vivo and in vivo has involved the use of macromolecules, such as horseradish peroxidase, lactulose, mannitol, or $^{51}$Chromium-EDTA (Draper et al., 1983; Bjarnason et al., 1984; Keshavarzian et al., 1994, 2001). In this study, we employed intra gastric administration of exogenous LPS for determination of intestinal permeability because increased permeability to molecular probes, such as horseradish peroxidase, EDTA, or sugars in the small intestine is not entirely indicative of increased permeability to endotoxins. A complicating issue regarding the use of LPS as a marker of increased intestinal permeability is that LPS may cause increases in permeability by itself (Garcia Soriano et al., 2001). We examined dose-response effects of LPS on intestinal permeability and found that high doses of LPS ($\geq$5 mg/kg b.w.t.) increased plasma endotoxin levels significantly. According to our data, administration of 1 mg/kg LPS alone did not cause an increase in intestinal permeability compared with untreated controls. More importantly, acute ethanol exposure caused significant increases in intestinal permeability to endotoxin, as illustrated by the increased level of plasma endotoxin detected in mice treated acutely with ethanol and loaded with LPS as compared with animals treated with ethanol alone. This result suggests that the endotoxemia observed in our work was a result of acute ethanol-induced increase in intestinal permeability to endotoxin and that zinc inhibition of circulating plasma endotoxin is due to preservation of intestinal barrier function.

Although the results of this study provide evidence that zinc supplementation had potent inhibitory effects on acute ethanol-induced liver injury via prevention of intestinal damage, there are questions and limitations that need to be addressed pertaining to the mechanisms in which zinc provides this protection, and in particular, whether these effects are due to zinc action alone. In previous studies, we have shown that zinc supplementation ameliorates cell damage in the liver parenchyma, and this was associated with inhibition of oxidative stress (Zhou et al., 2002). More recently, we found that acute ethanol-induced hepatocyte cell death mediated through the Fas (CD95/Apo-1/Fas) ligand pathway was significantly inhibited by zinc pretreatment (Lambert et al., 2009). In addition, zinc is well known to be protective in cells of the macrophage-monocyte lineage, although the mechanism is still not clear (Brown and Carter, 1990). Therefore, although we observed a correlation between the protective effect of zinc against TNF-α production and the inhibition of acute alcohol-induced increases in endotoxin absorption at the level of the intestine, we cannot rule out direct protection of Kupffer cells by zinc. Intestinal absorption of zinc is primarily dependent on metallothionein, and this zinc-binding protein has been shown in many experimental systems to have significant protective effects (Cunningham-Rundles et al., 1999; Takano et al., 2000). Further studies are required to delineate whether the inhibitory actions by zinc at the intestinal mucosal layer are independent of metallothionein. Last, we cannot exclude the possibility that zinc administered intragastrically might have a protective effect on intestinal microflora, leading to a decrease in endotoxin release in the lumen of the intestine during acute ethanol challenge.

In conclusion, this study demonstrated that zinc is directly involved in inhibition of acute alcohol-induced liver injury and that this protection entails prevention of endotoxemia through preservation of intestinal barrier function. The significance of this finding is that dietary zinc supplementation inhibits acute ethanol-induced liver injury at different levels and that an important site of zinc protection may be in the intestine.

References


**Address correspondence to:** Dr. Y. James Kang, Department of Medicine, University of Louisville School of Medicine, 511 S. Floyd St., MDR 530, Louisville, KY 40202. E-mail: yjkang01@athena.louisville.edu